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Trophic transfer of differently functionalized
zinc oxide nanoparticles from crustaceans
(*Daphnia magna*) to zebrafish (*Danio rerio*)

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Abstract (294 words)

The potential uptake and trophic transfer of nanoparticles (NP) is not well understood so far and for ZnO NP the data presented in peer-reviewed literature is limited. In this paper the influence of surface functionalization on the uptake and depuration behavior of ZnO NP, ZnO-OH NP and ZnO-octyl NP in *D. magna* was studied. Bulk ZnO particles ($\leq 5\mu\text{m}$) and ZnCl_2 were used as references for uptake of particles and dissolved species of Zn, respectively. Furthermore, the trophic transfer of ZnO NP and ZnO-octyl NP from daphnids (*Daphnia magna*) to zebra fish (*Danio rerio*) was studied. For ZnO NP and ZnO-octyl NP fast uptakes in *D. magna* were observed, whereas no measurable uptake took place for ZnO-OH NP. Lower body burden of ZnCl_2 was found compared to both ZnO NP and ZnO-octyl. Contrary, the body burden for bulk ZnO was higher than that of ZnO NP but lower than ZnO-octyl. The higher body burdens found for functionalized ZnO-octyl NP than for non-functionalized ZnO NP showed that the functionalization of the NP has a high influence on the uptake and depuration behavior. Though no mortality was observed, the resulting body burdens were 9.6 times (ZnO NP) and 47 times (ZnO-octyl NP) higher than toxic levels reported for zinc in *D. magna*. Consequently, the zinc recovered in the animals was not solely due to soluble zinc, but agglomerates/aggregates of ZnO NP or ZnO-octyl NP contributed to the body burdens. The trophic transfer study showed uptake of both ZnO NP and ZnO-octyl NP reaching more than tenfold higher levels than those obtained through aqueous exposure in other studies. This study contributes to expand the available data on uptake behavior of differently functionalized ZnO NP in *D. magna* and the potential trophic transfer from zooplankton to fish.

Keywords: Biomagnification; Nanoecotoxicology; ZnO nanoparticle; Coating; Uptake kinetics; Depuration kinetics

1. Introduction

Zinc oxide nanoparticles (ZnO NP) are now commonly used in a range of consumer goods like personal care products (Serpone et al., 2007), paints (Cai et al., 2006), and anti-corrosion agents (Ramezanzadeh and Attar, 2011). The production volume of ZnO NP was estimated to approximately 510 tons/year in the United States (Gottschalk et al., 2009) and the diverse range of uses makes release to the environment inevitable. Multiple studies have documented the ecotoxicity of ZnO NP (Franklin et al., 2007; Heinlaan et al., 2008; Aruoja et al., 2009; Wiench et al., 2009; Bai et al., 2010; Blinova et al., 2010; Fabrega et al., 2011; Shaw and Handy, 2011) much less attention has been paid to the potential uptake of ZnO NP by aquatic organisms. This trend is not unique for ZnO NP as in general little is known about uptake and depuration behavior of engineered nanomaterials. For gold NP some uptake has been shown in *Daphnia magna* (Lovern et al., 2008; Skjolding et al., 2014) as well as in filter-feeding bivalves (*Corbicula fluminea*) (Hull et al., 2011), but uptake beyond the intestine was not observed in these studies. A similar observation was made for TiO₂ NP aggregates (>200 nm) (Galloway et al., 2010) whereas Rozenkranz et al. (2009) showed uptake and translocation of polystyrene beads (20 nm) from the gut and into lipid droplets of *D. magna*. Uptake past the gut was also observed by exposing ellipsoid quantum dots (QD) (12 nm by 6 nm) to rotifers (Holbrook et al., 2008). For ZnO NP, Hao et al. (2013) recently found that ZnO NP significantly accumulated and distributed in various tissues of juvenile carp (*Cyprinus carpio*). Only very few studies have reported on trophic transfer of engineered NPs; Zhu et al. (2010b) demonstrated transfer of TiO₂ NP from *D. magna* to *D. rerio* and studies using QD found evidence of potential trophic transfer (Holbrook et al., 2008; Jackson et al., 2012). However, as identified by Menard et al. (2011) and Ma et al. (2013) there is still a substantial lack of data describing the bioaccumulative behavior of nanoparticles and their potential trophic transfer.

Most uptake studies have used pristine, uncoated, and non-functionalized NPs like Au NP, TiO₂ NP, and ZnO NP (see recent reviews by Menard et al. (2011) on TiO₂ NP and Ma et al. (2013) on ZnO NP). The fate and behavior of NP in environmental media may be strongly dependent on the NP functionalizations since these will influence the surface charge, solubility, aggregation behavior, and suspension stability. Concurrently, these factors may influence the biological uptake of NP (Tejamaya et al., 2012; Siebein et al.,

2013). For example, Siebein et al. (2013) showed 1.7 and 2.7 times higher uptake of negatively charged and neutral QDs, respectively, compared to positively charged QD. Evidently it is important to test the influence of differently functionalized NP on their uptake behavior and possible trophic transfer, however, to the best of our knowledge, this has not been done yet. For functionalized carbon nanotubes an alteration was observed upon ingestion by *D. magna* (Roberts et al., 2007). Hence, changes in the uptake patterns may depend on both the trophic level (Siebein et al., 2013) and the NP functionalization in question. It may be expected that NP functionalized with hydrophilic groups will be taken up to a lesser extent than NP functionalized with lipophilic groups. In this paper we put forward the following null hypothesis: Functionalization of ZnO NP cause no differences in uptake or trophic transfer patterns observed in pelagic aquatic organisms.

This hypothesis was tested using three types of functionalized ZnO NP (pristine ZnO NP, functionalized ZnO-OH NP, and ZnO-octyl NP) with identical primary sizes in a series of uptake studies with *D. magna* as test organism. This organism was chosen due to its high ecological importance, widespread use in guideline laboratory tests, and due to its feeding trait that results in a high potential for uptake of particles from the water column. Another series of experiments aimed at quantifying the trophic transfer of ZnO NP were performed by feeding zebra fish (*D. rerio*) with *D. magna* exposed to ZnO NP prior to the feeding experiments.

2. Materials and Methods

2.1 Chemicals and characterization

Nanoparticle (ZnO, ZnO-OH and ZnO-octyl) powders were received from L'Urederra (Los Arcos, Spain) and characterized by means of Dynamic Light Scattering (DLS), Transmission Electron Microscopy (TEM) and zeta-potential. The purity was analyzed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). All characterization was carried out at a concentration of 1.0mgZn/L. For DLS and zeta-potential the powders were dispersed in MilliQ water. Additionally, size distribution (DLS) and suspension stability (zeta-potential) of all ZnO NP in Elendt M7 medium were determined on a Malvern, Zetasizer (Malvern, Zetasizer

Nano-ZS) at 20°C using a backscattering angle of 173°. Each determination was done in triplicates with 30 measurement runs of 1mL sample solution in 1 x 1cm plastic cuvettes. Stokes-Einstein equation was used to calculate the hydrodynamic diameter of the NP (ZnO, ZnO-OH, ZnO-octyl) using the cumulant method for fitting the autocorrelation function (Kretzschmar et al., 1998).

Stock solutions of 50mgZn/L of ZnCl₂ and suspensions of 50mgZn/L ZnO NP were prepared in the ISO 6341 medium (294mg/L CaCl₂·2H₂O, 123mg/L MgSO₄·7H₂O, 64.8mg/L NaHCO₃, 5.8mg/L KCl) (ISO, 2012) for acute toxicity tests with *D. magna* and in Elendt M7 medium (content of macro-ions: 294mg/L CaCl₂·2H₂O, 123mg/L MgSO₄·7H₂O, 64.8mg/L NaHCO₃, 5.8mg/L KCl) (OECD, 2008) for uptake, depuration and trophic transfer studies with *D. magna* and *D. rerio*. Upon addition of ZnO NP and ZnO-OH NP the dispersions were sonicated for 15min at 40kW with a 13mm disruptor horn in an ice cooled beaker. For sonication a total volume of 300mL was used. After sonication the dispersions appeared cloudy white. For preparation of the 50mgZn/L ZnO-octyl NP stock solution the powder was wetted with 0.03mL acetone in a 300mL measuring flask and ISO 6341 medium was added. This suspension was then sonicated as described above. A solvent control was prepared in the same manner with omission of Zn-octyl NP.

2.2 Acute toxicity tests with *D. magna*

The acute toxicity tests were carried out in accordance with ISO:6341 (ISO, 2012). Stock solutions/suspensions of test compounds were diluted with ISO medium in 100mL measuring flasks to the following concentrations for ZnO NP: 50, 25, 10, 4.0, 2.0, and 1.0mgZn/L and for ZnCl₂: 5.0, 2.0, 1.0, 0.5, 0.2, and 0.1mgZn/L. The ISO medium and concentrations in the dilution series were adjusted to a pH of 7.8 ± 0.2 by drop wise addition of 0.1M HCl or 0.1M NaOH. The appearance of the dispersions varied from cloudy white at 50mg/L to transparent at concentrations ≤4mg/L. At each concentration level four replicates containing 25mL in 50mL glass beakers were used. Five living *D. magna* neonates (<24 hours old) were transferred to each replicate with a fine masked net. The beakers were placed at 20 °C in the dark and after 24 and 48 hours the number of dead and living neonates was counted. Dead neonates were defined as

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5 animals that did not move after gently prodding. Oxygen concentrations and pH were measured to ensure
6 that the validity criteria of the tests were fulfilled (8.6-8.9mgO₂/L and pH 7.6-7.9).
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10 11 2.3 Uptake and depuration study with *D. magna* 12

13 Uptake and depuration studies with ZnO NP and *D. magna* were performed in 50mL glass beakers filled
14 with 25mL of 1.0mgZn/L suspensions of ZnO NP prepared from the 50mg/L stock suspensions as described
15 above. Tests were prepared with three replicates each containing five *D. magna* (4-5 days old) for each time
16 interval used. The beakers were incubated at 20°C in the dark and one set of three replicates was sacrificed
17 after 30min, 1h, 2h, 4h, and 24h. After 24 hours all living daphnia were transferred to a series of 50mL
18 beakers filled with 25mL of clean Elendt M7 media for the depuration studies. Three replicates of five
19 animals were sacrificed after 10min, 30min, 1h, 4h and 24h. In parallel two additional treatments were
20 prepared with ZnCl₂ and ZnO bulk at same test conditions, but test organisms were only sacrificed after 24
21 hours of uptake.
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34 Prior to chemical analysis the test organisms were transferred to clean Elendt M7 and immediately hereafter
35 to 5mL of 7M HNO₃ and left for acid digestion at room temperature overnight. After digestion, 15mL of
36 distilled water was added and the vials were stored at room temperature until chemical analysis.
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42 2.4 Trophic transfer study with *D. magna* and *D. rerio* 43

44 The zebrafish used for the trophic transfer tests were spawned and cultivated in the laboratory (DHI,
45 Hørsholm, Denmark). Zebrafish were approximately 3 months old when used for testing (dry weight
46 15±4.9mg per individual). One week prior to testing the zebrafish were acclimatized in 1L beakers filled
47 with 500mL Elendt M7 media. A light:dark cycle of 14:10 hours was used for the acclimatization period as
48 well as for the tests. The fish were fed with *D. magna* (4-5 days old, dry weight 88±9µg/organism) at a daily
49 rate of approximately 8% of the wet weight of the fish. For the uptake tests, the fish were fed with *D. magna*
50 (4-5 days old) that had been exposed to ZnO NP and ZnO-octyl NP at a concentration of 1.0mgZn/L for 24
51 hours. The amount of Zn in *D. magna* used for feeding was determined by ICP-OES analysis of five *D.*
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5 *magna* to day 1, 3, 5, 7, 10 and 14. Every day at the same time daphnia were added to the test beakers and
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7 the zebrafish were allowed to eat for 2 hours. The numbers of daphnia left in the test beakers after the 2
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9 hours were noted and corrected for in the analysis of the results. Every day the pH and O₂ content was
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11 measured. Afterwards the media was renewed by gently pouring the media and the fish into a beaker with a
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13 net on the top to ensure the fish would always be submerged in water. The test beaker was then flushed with
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15 distilled water and 500mL of fresh Elendt M7 medium was added before the fish were gently returned to the
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17 test beaker. This was repeated daily for all the beakers to minimize the uptake of additional Zn from the
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19 aqueous phase due to excretion from the test organisms. On day 1, 3, 5, 7, 10 and 14 three beakers were
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21 sacrificed to measure the uptake of ZnO NP and ZnO-octyl NP, respectively. This was done by pouring the
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23 fish and media into a beaker with a net on the top. The fish was then transferred to a separate tank with
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25 Elendt M7 and the anaesthetic compound MS-222 (Ethyl 3-aminobenzoate methanesulfonic acid). The MS-
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27 222 was dosed to induce euthanasia within <30sec of exposure. The fish were then transferred to Teflon
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29 bottles which were weighted prior to the transfer. Afterwards, the Teflon bottles were put in an oven at
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31 105°C until no change in weight was to be observed. The dry weight of the fish was then noted to be used for
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33 further analysis. 5mL of 7M HNO₃ was added and the lid was carefully tightened before digestion. The
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35 samples were digested for 20min at 140°C in an autoclave. After digestion the sample was diluted with
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37 15mL of distilled water.
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42 *2.5 Chemical analysis*

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45 Chemical analysis of zinc was carried out with ICP-OES (Varian Vista-MPX CCD simultaneous ICP-OES)
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47 using the following settings: internal max standard error $\pm 15\%$, scanning with internal standard yttrium at
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49 wavelengths 377nm and 433nm, the standard for Zn was at wavelengths 203nm, 206nm and 214nm. The
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51 standard which showed the clearest signal was used for quantification. Furthermore, a test for medium
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53 interference with the measurements was performed (standard addition test). For all the samples the sample
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55 matrix did not show an interference of more than $\pm 15\%$. The limit of detection for dissolved zinc salt and
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57 ZnO-NPs was 1.4 μ g/L.
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2.6 Data analysis

For the analysis of acute toxicity data the program ToxCalc™ v5.0 was used for estimation of LC-values and corresponding 95% confidence intervals. This was done by linear regression on probit transformed data using maximum likelihood estimation for the point estimations (Tidepool Scientific). One-way ANOVA ($p < 0.05$) with a Tukey's HSD test was used to test for significant differences of data found in the uptake, depuration and trophic transfer studies (GraphPad Prism v5.0). To evaluate the uptake and depuration studies, rates for the initial uptake ($k_{1,initial}$) and depuration ($k_{2,initial}$) were modelled using first-order rate model given in Eq. 1 using non-linear curve fitting (GraphPad Prism v5.0).

$$C_t = \frac{C_w k_u}{k_e} (1 - e^{-k_e t}) \quad (1)$$

Where C_t is the concentration in the organism at time t , C_w is the water phase concentration or the concentration in the food for the trophic transfer study, k_u is the uptake rate and k_e is the elimination rate.

3. Results

3.1 Characterization of ZnO, ZnO-OH, and ZnO-octyl NP

The TEM images of the powder ZnO NP shows the particles' shapes and sizes, which in general were found to be homogenous throughout the samples (Supplementary Figure S1). The initial DLS measurements of both pristine and functionalized ZnO NP in MilliQ showed stable particle suspensions with zeta-potentials of 19.91 ± 4.69 mV, 33.8 ± 14.5 mV and 29.2 ± 6.3 mV for ZnO NP, ZnO-OH NP, and ZnO-octyl, respectively (Table 1). The size distribution data showed a trend of ZnO NP having the smallest hydrodynamic diameter, followed by ZnO-OH NP, and ZnO-octyl NP (Table 1). The characterization carried out on suspensions in Elendt M7 medium showed similar size of ZnO-OH NP and ZnO-octyl, while ZnO NP were smaller, however the hydrodynamic diameters increased markedly after both 0 and 24 hours in suspension (Table 1). Significant decreases in suspension stabilities were observed for all ZnO NP immediately upon transfer to Elendt M7 medium as evidenced by measurements of zeta potentials as well as visual observations. Further decreases in stability were observed from 0 to 24h with all ZnO NP being in the unstable range from -10mV

to 10mV after 24h (Table 1). After 24h in Elendt M7 medium all suspensions were found to be very heterogeneous with polydispersity index (PDI) close to 1.

3.2 Acute toxicity of zinc chloride and zinc nanoparticles in *D. magna*

The acute toxicity tests showed LC_{10, 48h}-values with corresponding 95% confidence intervals of 0.14mgZn/L [0.08;0.19], 0.88mgZn/L [0.48;1.2], 6.9mgZn/L [3.6;9.7], and 4.1mgZn/L [2.3;5.9] for ZnCl₂, ZnO NP, ZnO-OH NP, and ZnO-octyl NP, respectively. LC_{50, 48h}-values with corresponding 95% confidence intervals were found to be 0.41mgZn/L [0.31; 0.53], 1.9mgZn/L [1.5; 2.4], 15.5mgZn/L [11.4; 20.9], and 13.7mgZn/L [10.1; 18.9] for ZnCl₂, ZnO NP, ZnO-OH NP, and ZnO-octyl NP, respectively. The lowest LC_{10-48h}-value found for the nanoparticles (0.88mg Zn/L [0.48; 1.2] for ZnO NP) formed the basis for the 1.0mgZn/L exposure concentrations applied in the uptake and depuration studies.

3.3 Uptake and depuration studies in *D. magna*

In the 24h uptake and 24h depuration studies of ZnO NP, ZnO-OH NP, and ZnO-octyl NP with *D. magna* no death or perishing of organisms was observed. The data presented in the following sections were corrected for background levels of Zn in control animals (background in animals 223±65mgZn/kg dry weight, n=9). Figure 1a (closed symbols) shows that the content of ZnO NP in *D. magna* increased during the first four hours of exposure. Between 4 and 24 hours the concentration remained stable (no statistically significant difference, p<0.05) as indicating by the concentration plateau reached after 4 hours. The body burden of Zn in *D. magna* after 24h was 7690±3580mgZn/kg dry weight. The depuration of Zn after transfer of *D. magna* to clean medium revealed a rapid decrease in Zn-content within the first 10min after transfer of organisms to clean medium (Figure 1a, open symbols).. For both the uptake phase and the depuration phase the Zn concentrations reached after 10min were significantly different (p<0.05) from those of the controls. The decrease in the content of Zn in animals in the depuration phase leveled out after 30min, however depuration continued, as evidenced by the significantly lower content measured after 24h compared to preceding measurements. It should, however, be noted that after 24h of depuration the level of Zn in the organisms was

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5 still significantly higher ($p<0.05$) than background level in the organism (Figure 1a). The modelled uptake
6 and depuration rates are shown in Table 1.
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11 The uptake and depuration of ZnO-octyl NP is shown in Figure 1b. It is seen that the concentration of ZnO-
12 octyl NP increased until 4h of exposure. Between 4 and 24h the body burdens remained stable (no
13 statistically significant difference, $p<0.05$). The body burden after 24h was $37230\pm2560\text{mgZn/kg dry weight}$.
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15 After transfer to clean medium a rapid depuration was observed from 10 to 30min. After 4h of depuration
16 this decreasing trend leveled off, but even after 24h the body burdens of exposed animals were significantly
17 higher ($p<0.05$) than that of the control animals. The modelled uptake and depuration rates are shown in
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19 Table 1.
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28 For ZnO-OH NP no statistically significant differences ($p<0.05$) between the Zn contents of exposed and
29 non-exposed animals were observed during the 24 hour uptake study (Figure 1c). The body burden achieved
30 was $287\pm91\text{mgZn/kg dry weight}$ (Table 2). Due to no apparent uptake of ZnO-OH NP in *D. magna* no study
31 on trophic transfer from *D. magna* to *D. rerio* was performed with ZnO-OH NP.
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38 3.4 Trophic transfer studies with *D. magna* and *D. rerio*

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40 The trophic transfer of ZnO NP and ZnO-octyl NP from *D. magna* to *D. rerio* was assessed by feeding pre-
41 exposed *D. magna* to *D. rerio* during a 14 days uptake period. This was followed by 7 days depuration
42 period in which the fish were fed with non-exposed *D. magna*. All concentrations reported are corrected for
43 background concentration ($242\pm20\text{mgZn/kg}$, $n=3$). All pH-values and O_2 concentrations measured at the end
44 of tests were in compliance with the guideline for bioconcentration tests with *D. rerio* (OECD 2012). During
45 the course of the experiments no fish mortality was observed. Consequently, the data in the following
46 sections were only corrected for weight of each individual organism. In order to minimize the use of test
47 animals and since the body burden in *D. magna* resulting from ZnCl_2 exposure was significantly lower than
48 for both ZnO NP and ZnO-octyl NP, no test with the ZnCl_2 control was carried out.
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Figure 2a shows the uptake of ZnO NP through trophic transfer from *D. magna* (feed) to *D. rerio* (predator) during 14 days of feeding with exposed *D. magna*. The body burdens of the fish steadily increased until day 5 where it leveled off. The measurements performed on fish exposed for more than five days were neither statistically different from each other nor from the level found after five days ($p < 0.05$). Thus, a body burden of $890 \pm 180 \text{ mg Zn/kg dry weight}$ ($n=12$) was calculated by combining all measured data past day 5 (Table 2). Figure 2a (14-21d) shows the content of Zn in animals exposed to ZnO NP after shifting the feeding to non-exposed *D. magna*. No depuration was observed during the first day (day 14 to 15), but a gradual decrease in body burden was observed during the first three days of depuration (until day 17) after which the body burden in the fish leveled out. The modelled uptake and depuration rates are shown in Table 2.

Figure 2b shows the uptake of ZnO-octyl NP in *D. rerio* during 14 days of feeding with exposed *D. magna* and 7 days of depuration feeding with non-exposed *D. magna*. It is seen that the body burden slowly increased during the first three days of exposure. From day 3 to 5 the increase was steeper but increased linearly between day 5 and 14. Thus, the body burden did not reach a constant level during the 14 days of feeding with pre-exposed *D. magna*. The body burden reached at day 14 was $2170 \pm 410 \text{ mg Zn/kg dry weight}$ ($n=3$). The data obtained in the depuration study of fish exposed to *D. magna* fed with ZnO-octyl NP, Figure 2b (day 14 to 21), shows a rapid depuration immediately after the fish were fed with non-exposed daphnia. The measurements performed between day 14 and 19 indicate that no plateau was reached within the tested time frame. Modelled uptake and depuration rates are shown in Table 2.

4. Discussion

In the present study it was found that the functionalization of the ZnO-containing nanoparticles affected their uptake in *D. magna* (Figure 1). While ZnO NP and ZnO-octyl NP showed similar trends of a rapid initial uptake during the first four hours of exposure, no uptake of ZnO-OH NP could be determined throughout the whole exposure period (24h) (Figure 1c). These results indicate that differently functionalized NPs exhibit different bioavailability to *D. magna* even though the core material is the same. In a study by Larner et al. (2012), using radio labelled ZnO NP and sediment dwelling amphipods (*Corophium volutator*), it was found

that the uptake of ZnO NP, bulk Zn, and ZnCl₂ did not differ significantly when results were expressed in terms of bioconcentration factor (BCF) for the whole organism. However, it should be noted that the use of BCF for nanoparticles on a whole organism level is inappropriate since the basic assumptions and estimation models behind BCF may be violated when particles are taken up and excreted through different mechanisms than for the dissolved chemicals for which BCF is defined (Handy et al., 2012). Contrary to the study by Larner et al. (2012) this study revealed different uptake of non-functionalized ZnO NP compared to those of both ZnCl₂ and ZnO bulk, i.e. 4.6 times higher and 2.3 times lower body burdens, respectively. While dissolution may play a role in the uptake pattern observed for ZnO particles it is more likely that agglomerates contribute more in explaining the higher uptake of bulk ZnO and ZnO NP compared to that of ZnCl₂ (Table 2). For ZnO-OH NP we did not observe any measurable uptake, while ZnO-octyl NP was taken up 4.8 times more than ZnO NP suggesting that the functionalization does influence the uptake.

The body burden found in the study by Larner et al., (2012) in a sediment dwelling organism (*C. volutator*) of 87mg Zn/kg after exposure to ZnO NP is 90 and 430 times lower than the body burdens found in *D. magna* in the present study for ZnO NP and ZnO-octyl NP, respectively (Table 2). This difference may partly be explained by the use of higher aqueous exposure concentrations in the present study (approximately 48 times higher than in Larner et al., 2012). The high body burden observed in our study indicates that even though the processes of aggregation and sedimentation occurs rapidly at high ionic strength and at neutral pH (Bian et al., 2011), pelagic organisms are still subject to uptake of NP. High uptake was also observed in *D. magna* for TiO₂ NP at exposure conditions (1mg/L TiO₂ NP in OECD reconstituted culture media) similar to this study (Zhu et al., 2010a). However, in contrast to the findings for ZnO NP, Zhu et al., (2010a) did not observed rapid depuration within the first hours of the depuration phase. While TiO₂ NP is not likely to dissolve in test medium, the opposite is the case for pristine ZnO NP e.g. Bian et al. (2011) and Larner et al. (2012). Thus, the uptake and depuration found in our study might be in the form of soluble Zn-species formed in the medium or the gut of *D. magna* after ingestion. Soluble Zn-species have been observed to undergo rapid uptake and depuration in *D. magna* (Muyssen and Janssen, 2002) and this may contribute to explain the difference in depuration patterns for ZnO NP and TiO₂ NP. However, since the optimal level of

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5 Zn in daphnia were reported in the range of 200-300mgZn/kg dry weight with toxic effects occurring at >800
6 mgZn/kg dry weight (Muyssen and Janssen, 2002) it is not plausible that all the uptake found in our study is
7 related to dissolved Zn-species, when considering that the body burdens of ZnO-octyl NP markedly
8 exceeded that of ZnCl₂ (Table 2). Despite the high body burden no lethality was observed for ZnO NP and
9 ZnO-octyl NP after 24 hours of exposure.
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17 For tests with selective filter feeders like *D. magna* the size of the NP could be a key parameter determining
18 the particle uptake. Studies have shown that the selective filtration for *D. magna* peak at around 500nm for
19 experiments carried out with spherical plastic beads ranging from 100nm to 3500nm (Gophen and Geller,
20 1984). Considering the sizes of ZnO NP, ZnO-OH NP, and ZnO-octyl NP in the medium used in the present
21 study (Table 1) it is possible that larger agglomerates formed were selectively filtered by *D. magna* from the
22 water column. Though a combination of soluble, complexed Zn-species, and ZnO NP as particles may
23 contribute to the uptake and depuration behavior observed in this study, the uptake of particles and
24 aggregates contribute significantly to the overall uptake observed (Table 2).
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36 For both ZnO NP and ZnO-octyl NP rapid initial depuration patterns were observed (Figure 2). The rate of
37 depuration decreased after 30min and more or less constant body burdens were reached. After 24h of
38 depuration, the level was still significantly elevated ($p<0.05$) compared to unexposed *D. magna*. This
39 indicates that ZnO NP and ZnO-octyl NP are available for trophic transfer even after 24 hours of depuration
40 in clean medium. Similar results were observed for TiO₂ NP after a 24h depuration period (Zhu et al., 2010a).
41 The fast initial depuration of ZnO NP and ZnO-octyl NP and the reported gut retention times of 3-60min in
42 *D. magna* (Peters and de Bernardi, 1987) suggest that the majority of the NP were not taken up past the gut.
43 However, the residual body burden exceeds the toxic level for Zn in *D. magna* as reported by Muyssen and
44 Janssen (2002). With a difference in residual body burden exceeding a factor of 10 at the end of depuration
45 period it is clear from this study that the different functionalization of NP affect the depuration pattern in *D.*
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The trophic transfer study showed that ZnO NP and ZnO-octyl NP were available for trophic transfer to *D. rerio* preying on pre-exposed living *D. magna*. Uptake of ZnO-octyl NP was observed after >3 days exposure and for ZnO NP all measurements made after day 1 showed body burdens higher than that of the unexposed controls (Figure 2). Steady state was reached for ZnO NP within the exposure period of 14 days while this was not the case for ZnO-octyl NP (Figure 2). Body burdens as high as 880 ± 180 and $2170 \pm 410 \text{ mg Zn/kg}$ dry weight were found for ZnO NP and ZnO-octyl NP, respectively (Table 2). Estimation of uptake through aqueous exposure to 10 mg Zn/L for 96h showed levels around 40 and 45 mg Zn/kg dry weight for ZnO NP and bulk ZnO respectively (Yu et al., 2011). These levels were approximately 20 and 50 fold lower than observed in this study for ZnO NP and ZnO-octyl NP, respectively. Though the exposure time was also shorter than the one used in this study, even at similar times of exposure (Figure 2) the concentration observed in our study was approximately 10 fold larger for both tested NPs than those observed by Yu et al. (2011). The clear difference found between the observed body burdens are most likely related to higher dietary uptake in the present study compared to uptake through aqueous exposure. Others studies have found values similar to ours for aqueous exposure and concluded that ZnO NP was likely to have low bioavailability thus presenting low hazard to non-benthic fish types (Johnston et al., 2010). Conversely, our study shows that even with low bioavailability of ZnO NP in aqueous suspension, ZnO NP is bioavailable through dietary uptake yielding high body burdens (Table 2). It has previously been emphasised that nanomaterials should be tested through the most likely route of exposure (Ryman-Rasmussen et al., 2009) and earlier studies have identified uptake of a range of different NP through diet in aquatic organisms (QD: Holbrook et al. (2008), Jackson et al. (2012); TiO_2 NP: Zhu et al. (2010b); ZnO NP: Larner et al. (2012)). Consequently, this study demonstrates that dietary exposure should be regarded as an important route of uptake when assessing the risk of nanomaterials.

5. Conclusion

In the studies of uptake and depuration behavior of ZnO NP, ZnO-OH NP and ZnO-octyl NP in *D. magna* fast uptakes were found for ZnO NP and ZnO-octyl NP. For ZnO-OH NP no measurable uptake took place. Higher body burdens were found for functionalized ZnO-octyl NP than for the non-functionalized ZnO NP.

When comparing results obtained with ZnO NP, ZnO-OH NP and ZnO-octyl NP under identical exposure conditions it is concluded that the functionalization of the NP has a high influence on the uptake and depuration behavior. The resulting body burdens found in this study were 9.6 times (ZnO NP) and 47 times (ZnO-octyl NP) higher than toxic levels reported for Zn-salts in *D. magna*. Yet, in this study no mortality was observed in animals with these high body burdens of ZnO-containing nanoparticles. Consequently, the Zn recovered in the animals was not solely due to soluble zinc but agglomerates/aggregates of ZnO NP or ZnO-octyl NP contributed significantly to the body burdens. The trophic transfer study showed uptake of both ZnO NP and ZnO-octyl NP reaching values exceeding by tenfold the levels obtained through aqueous exposure in other studies.

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Declaration of interest

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Figure and table captions

Figure 1: 24 hours uptake (filled diamonds) and depuration (clear diamonds) with *D. magna* using 1.0mgZn/L of a) ZnO NP, b) ZnO-octyl and c) ZnO-OH in the uptake phase (mean \pm standard deviation; n=3). The model fit is indicated by the solid line.

Figure 2: 14 days of uptake (filled diamonds) and 7 days of depuration (clear diamonds) with *D. rerio* feeding on pre-exposed *D. magna* (1.0mgZn/L with ZnO NP (a), ZnO-octyl (b) for 24 hours) in the uptake phase and clean *D. magna* in the depuration phase (mean \pm standard deviation; n=3). The model fit is indicated by the solid line.

Table 1: Characterization of differently functionalized ZnO NP in MilliQ and M7 using parameters: purity, nominal size, hydrodynamic size and zeta-potential. In parentheses is stated the polydispersity index (PDI). For polydisperse samples (PDI > 0.2) the data should only be used for interpretation of trends in data. Actual hydrodynamic diameters cannot be determined.

Table 2: Results from uptake studies using *D. magna* exposed to ZnCl₂, ZnO bulk, ZnO NP and ZnO-octyl NP (1.0mgZn/L) for 24 hours and for *D. rerio* after 14 days uptake feeding on *D. magna* pre-exposed for 24h to 1.0mgZn/L of ZnO NP and ZnO-octyl NP, respectively. Depuration periods were 24 hours for *D. magna* and 7 days for *D. rerio*. Uptake and depuration rates were modelled using a first-order rate model. The R² is the correlation coefficient for the model fit. Numbers in parentheses are 95% confidence intervals of parameter estimates. All values are corrected for background content of Zn measured in clean animals. N/A: no data.

Supplementary figure and table captions

Figure S1: Representative TEM image and histogram of ZnO NP (30 \pm 17nm, n=894) characterized in ultrapure water.

Table 1

| Nanoparticles | Purity ^a [Zn %(w/w)] | Primary core size ^b [nm] | Hydrodynamic diameter ^c [nm] | | | | Zeta-potential [mV] | | | |
|---------------|------------------------------------|---|--|-------------|-------------|-------------|------------------------|----------|-----------|-----------|
| | | | MilliQ | | M7 | | MilliQ | | M7 | |
| | | | 0h | 24h | 0h | 24h | 0h | 24h | 0h | 24h |
| ZnO NP | 80.2 | 30 ± 17 | 111.8±24.9 | 165.0±26.4 | 770±220 | 2020±120 | 19.9±4.69 | 18.5±5.1 | -2.5±0.48 | -3.5±1.5 |
| | | | (0.31±0.08) | (0.28±0.06) | (0.38±0.05) | (0.91±0.07) | | | | |
| ZnO-OH NP | 56.6 | 30 ± 17 | 462.0±118.8 | 3600±1222 | 2420±730 | 6490±1660 | 33.8±14.5 | 13.5±1.4 | 23.3±1.3 | -0.37±4.3 |
| | | | (0.73±0.15) | (0.69±0.28) | (1) | (1) | | | | |
| ZnO-octyl NP | 54.0 | 30 ± 17 | 670.2±230.0 | 2533±1186 | 1990±440 | 5520±1220 | 29.2±6.3 | 23.1±6.6 | 19.6±1.3 | -8.0±0.6 |
| | | | (0.53±0.09) | (0.64±0.18) | (1) | (1) | | | | |

Data obtained by ^a ICP-MS; ^b TEM; ^c DLS in MilliQ water

Table 2

| <i>D. magna</i> | | | | | <i>D. rerio</i> | |
|--|-------------------|------------|--------------|---------------|-----------------|---------------------------|
| Parameters | ZnCl ₂ | ZnO bulk | ZnO NP | ZnO-octyl NP | ZnO NP | ZnO-octyl NP ^a |
| Body burden ^b | 1660±1020 | 17500±4300 | 7690±3580 | 37230±2460 | 890±180 | 2170±410 |
| [mg Zn/kg dw] | | | | | | |
| Uptake rate | N/A | N/A | 24500 | 38200 | 13 | 6.3 |
| [L kg ⁻¹ dw h ⁻¹] | | | (5410;43700) | (27300;49200) | (7.1;21) | (5;7.5) |
| Depuration rate | N/A | N/A | 3800 | 1100 | 15 | 5.8 |
| [h ⁻¹] | | | (570;7020) | (690;1410) | (6.1;24) | (1;11) |
| R ² | N/A | N/A | 0.32 | 0.75 | 0.67 | 0.87 |

^a A linear model was fitted to the data due to no plateau reached during uptake. Unit for uptake is [h⁻¹].

^b Average body burdens are shown as ±standard deviations.

Figure 1

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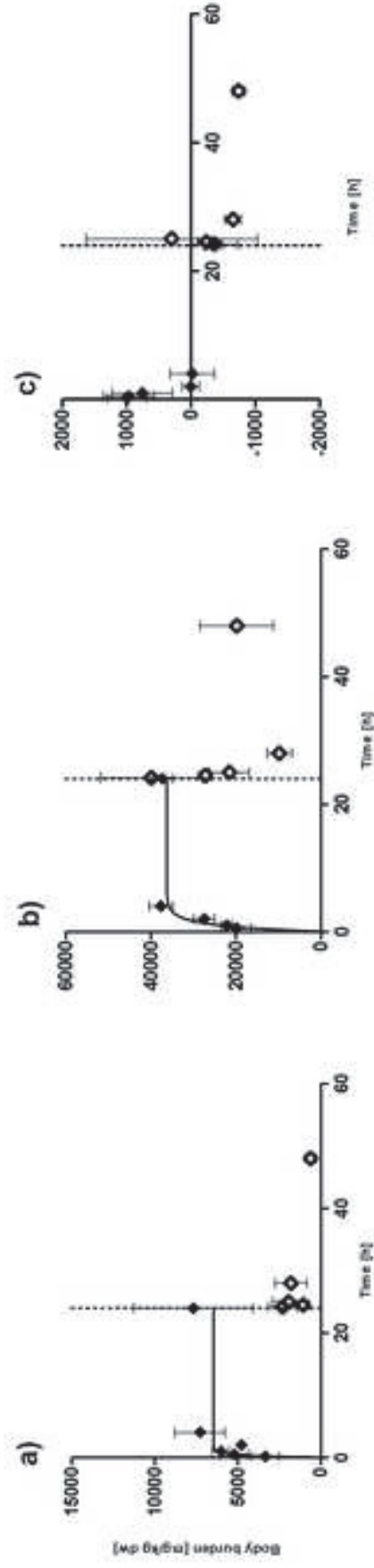
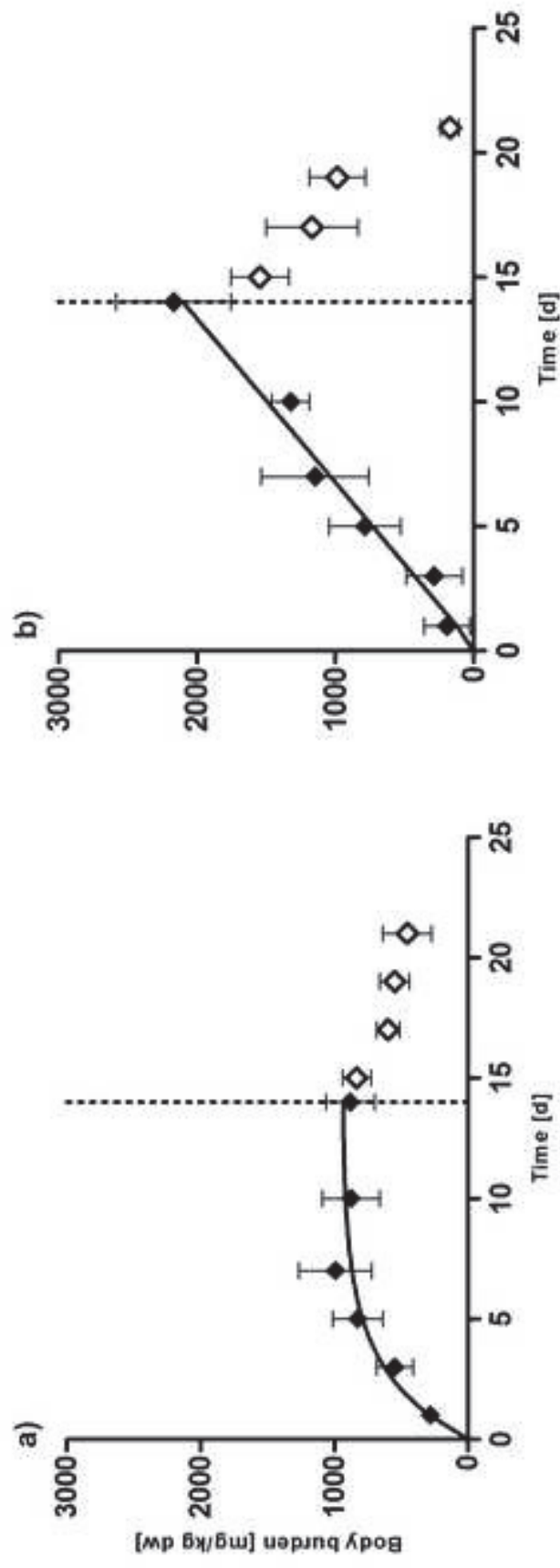


Figure 2

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Supplementary Material F1

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Highlights

- Surface functionalization of ZnO NP affected uptake and depuration rates in *D.magna*
- Zn content after exposure to functionalized ZnO NP differed from ZnCl₂ and ZnO bulk
- Trophic transfer of functionalized ZnO NP was observed from *D.magna* to *D.rerio*